

Assays for insulin, proinsulin(s) and C-peptide

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The first diabetic patient was treated with insulin in January 1922, revolutionizing the treatment of diabetes mellitus. The discovery and purification of insulin was the result of the collaborative work of Banting, Best, Collip and McLeod at the University of Toronto, a tale of discovery which makes fascinating reading.¹ The publication by Yalow and Berson² of a method for measuring insulin by radioimmunoassay (RIA) followed in 1959. Despite the enormous improvements and developments in immunoassay in the subsequent years, there remain a significant number of analytical and clinical problems to solve in the measurement of insulin and in our understanding of the pathogenesis of diabetes mellitus and hypoglycaemia.

SYNTHESIS OF INSULIN

Insulin is synthesized from its precursors, preproinsulin and proinsulin, in the β cells of the Islets of Langerhans.³⁻⁸ Within the β cell granules proinsulin is converted by a process of enzymatic cleavage to insulin and C-peptide (Fig. 1). Two endopeptidases, prohormone convertases 2 and 3 (PC2 and PC3), cleave the proinsulin molecule at two sites marked by pairs of dibasic amino acids. The type-1 endopeptidase (PC3) cleaves at the Arg³¹-Arg³² site at the junction of the B/C chains of proinsulin and the second endopeptidase (PC2) cleaves at the Lys⁶⁴-Arg⁶⁵ site, the proinsulin A/C junction. Both enzymes are calcium-dependent and have acidic pH optima, though their enzyme kinetics differ.¹⁰⁻¹² Through the action of carboxypeptidase H there is a loss of basic amino acids at each site to give the 'des' forms of the partially processed proinsulins. When the cleavage at both sites is complete, C-peptide and insulin are

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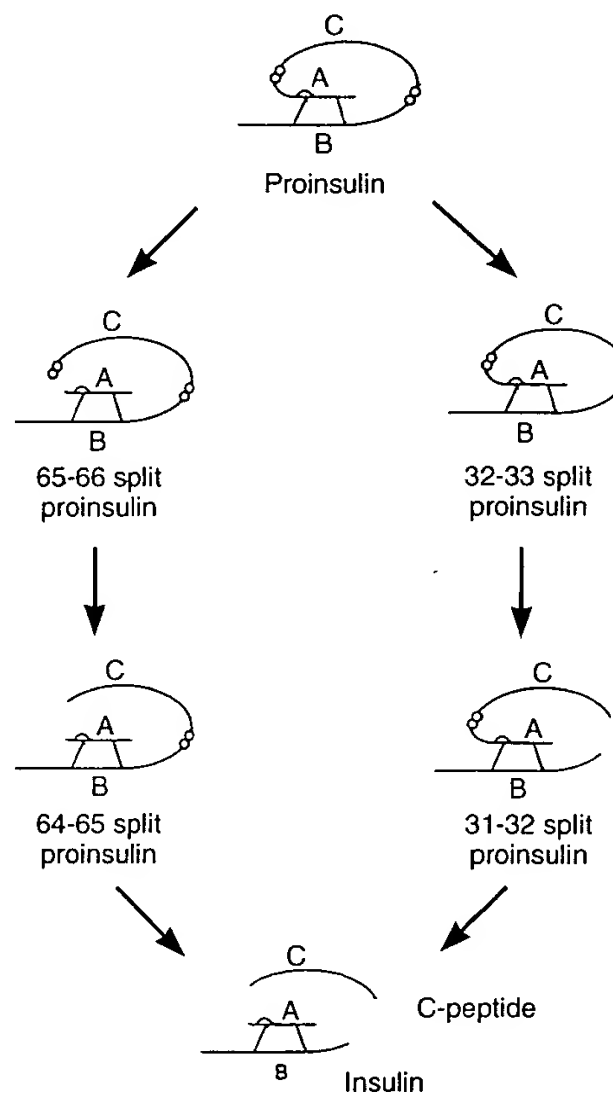


FIGURE 1. Processing of proinsulin to major intermediates and to insulin (letters identify chains, small circles represent the amino acid residues at positions 31 and 32 (Arg-Arg) and 64 and 65 (Lys-Arg). Copyright John Wiley and Sons Ltd, reproduced with permission.⁹

produced. Gel chromatography studies have shown heterogeneity of C-peptide in serum, which may arise either through *in vivo* metabolism and/or through degradation on storage.¹³ This has been confirmed by stable isotope dilution mass spectrometry.¹⁴ There is evidence that the des rather than the split forms are the major circulating form of the partially processed proinsulins, with the des 31,32 split proinsulin being present in higher concentrations in human

plasma than the des 64,65 split proinsulin. Insulin has the major biological activity though it has been suggested more recently that C-peptide and the partially processed forms of proinsulin may have biological activity.^{15,16}

The nomenclature used in the literature may not always be clear. In the early literature 'immunoreactive insulin (IRI)' was used to include those molecular species measured by the classic RIAs and this included insulin, proinsulin, the proinsulin conversion intermediates and any insulin derivatives produced by degradation, dimerization or glycosylation, for example. As will become apparent, this term may be misleading in that each species may not be recognized equally. More recently, the terms 'true insulin' and 'specific insulin' have been used where the hormone has been measured by a more specific, immunometric assay. To avoid confusion it is suggested that the terms 'immunoreactive' and 'true' insulin should no longer be used and that the specificity of the assay, with appropriate experimental details, should be given in the Methods sections of publications. Similarly, 'proinsulin' may be used where all proinsulin-like forms are measured. It is suggested that 'intact proinsulin' be used for the 86-amino-acid hormone and that the term 'partially processed (forms of) proinsulin(s)' be used for 32,33 split, des 32 split, des 31,32 split, 65,66 split, des 64 split and des 64,65 split proinsulins, depending on the specificity of the assays used and their natural occurrence. Careful reading of the method should indicate the extent to which each form is measured, although most immunoassays will not distinguish between the different forms of des and split proinsulins at the 32-33 site and similarly at the 65-66 site. The term 'total proinsulins' may be used to indicate the measurement of intact and partially processed proinsulins together. Whilst its meaning may be clear it should be remembered that the recovery of each form may not be 100%, nor equal for all forms.

STRUCTURE

Insulin is phylogenetically ancient, being found in mammals, reptiles, birds and fish. Its primary sequence has been determined and similarities between species demonstrated. It is a member of a larger family of molecules which all have some degree of homology in their sequence, for example, the insulin-like growth factors (IGF-I and IGF-II). C-peptide, in contrast, shows a greater degree of interspecies variability. Rats

and mice produce two different non-allelic proinsulins (I and II) which are processed to insulin I and insulin II and C-peptides I and II. These differences are important when considering assay specificity. There is some evidence that insulin can undergo non-enzymatic glycation *in vitro* and also in some animal models¹⁷ with a reduction in biological activity. The importance of this process in humans is not clear.

Having considered the synthesis and structure of the proinsulin(s) and insulin it becomes apparent that the main analytical challenge is the measurement of low concentrations of these peptides in the presence of molecules of similar structure.

CLINICAL USES FOR THE MEASUREMENT OF INSULIN, PROINSULIN AND C-PEPTIDE

Assays for insulin, proinsulin and C-peptide are used in two main areas of clinical interest. Firstly, in the investigation of hypoglycaemia, and secondly (principally for research purposes), in the investigation of the pathogenesis and treatment of diabetes mellitus. The analytical requirements for each cannot be assumed to be the same.

The differential diagnosis of hypoglycaemia in adults and children has been well reviewed.¹⁸⁻²¹ In essence the questions to be answered are: (a) if hypoglycaemia is present, is the (pro)insulin appropriately suppressed; and (b) if the insulin concentration is inappropriately elevated, is it endogenous insulin (i.e. is the C-peptide also elevated)? It has become clear since the discovery, by Steiner and Oyer in 1967,³ that islet cell adenomas secrete proinsulin, that the secretion of insulin by the tumour may be variable²² and that concentrations of circulating proinsulin may vary between patients.²³ Gorden and co-workers,²⁴ using a gel filtration method for measuring proinsulin and its intermediates (which they termed proinsulin-like component, PLC), found that 87% of insulinoma patients had a PLC of greater than 25% of the total immunoreactive insulin, although the reference range was not defined. It is not clear whether diagnostic accuracy is improved by measuring 'total' insulin, or insulin and/or proinsulin specifically. In the absence of such data there is an argument for using a non-specific insulin assay for the detection of insulinomas. The danger of using a specific assay for insulin is that the insulinomas secreting predominantly proinsulin will not be detected and the factitious

administration of animal insulin will be missed if a human-specific assay is used. The optimal investigation of 'hyperinsulinism' in neonates and children²⁵ and the associated analytical requirements are not so clear. There is evidence in neonates (both healthy term and pre-term infants) that the insulin/glucose relationship is significantly different from that in adults²⁶ and that there are significant elevations in proinsulin and 32–33 split proinsulin compared with adult circulating concentrations.²⁷

In the assessment and investigation of the pathogenesis of diabetes mellitus, data are accumulating that elevations of intact and partially processed proinsulins are markers of β cell dysfunction^{28–32} and may in some populations predict progression to type 2 diabetes mellitus.^{33–35} Defects in proinsulin processing may also be associated with diabetes mellitus (familial hyperproinsulinaemia)^{36,37} although not in all cases.³⁸ Thus, specificity considerations may determine that assays for insulin, intact proinsulin and partially processed proinsulins are needed to establish whether there is evidence for insulin resistance or insulin deficiency in type 2 diabetes.

The measurement of C-peptide may be used in both clinical situations, and its circulating concentrations are generally higher (nmol/L) than proinsulin (pmol/L). One particular use for the measurement of C-peptide is in the detection of residual β cell function in insulin-treated diabetics, generally for research purposes. In such instances, measurement of insulin or proinsulin will be of little use either because of exogenous administration of insulin or due to the possibility of anti-insulin antibodies being present in the patient's serum, which would interfere in the assay. Urinary C-peptide has been used, particularly for children, to assess residual β cell function. However, variations in urinary clearance of C-peptide under different physiological conditions and also in different disease states have been described. The use of urinary C-peptide measurement as a marker of insulin secretion should therefore be considered with caution.³⁹

Assays for these hormones, particularly insulin, are also used for forensic purposes and by the pharmaceutical industry, which may have particular requirements for assay performance.

SAMPLE REQUIREMENTS

The usual advice for collection of blood samples for measurement of insulin and C-peptide has

been for the blood to be collected (with or without anticoagulant), transported on ice, centrifuged immediately and for the serum/plasma to be stored frozen if assay is not possible in the next few hours. This advice derived from studies based on relatively imprecise radioimmunoassays and is still widely quoted. There has been a proliferation of blood collecting systems containing gel/bead separators and clot accelerators. The influence of these on results is not well documented and the use of these newer systems may mean that older advice on sample type may not be applicable. Smith and colleagues⁴⁰ found no difference between plain serum and gel serum collected using Vacutainer tubes (Becton Dickinson Vacutainer Systems, Meylan, France) for insulin, C-peptide and proinsulin analysis. In the case of commercially available assays, users should refer to the manufacturer's data sheet and for in-house assays these data should be established.

Insulin

In 1973 Feldman and Chapman⁴¹ showed that serum and plasma insulin were stable if whole blood was kept at room temperature for 4 h. However, these data referred to blood collected from only two subjects, with a limited range of insulin concentrations. More recently, Diver (personal communication) using the Pharmacia RIA for insulin (Pharmacia, Milton Keynes, UK) demonstrated that when whole blood from 12 individuals was kept at room temperature, the measured insulin concentrations (in the range 4–56 mU/L) were stable for up to 24 h and for up to 1 week at +4°C. Similar results were obtained using both an immunometric and an RIA for insulin but studying the stability of the hormone in whole blood only for a period of 4 h.⁴⁰ These workers also found no significant change in measured insulin when serum was stored for 2 days at +4°C. No effect of repeated freezing (–20°C) and thawing of the serum was demonstrated. Long-term stability studies are difficult to interpret, as the stability of the assay itself must be guaranteed. Feldman and Chapman,⁴¹ using a double antibody RIA, have suggested that there is a significant decrease in insulin concentration after storage at –20°C for 28 months. This requires confirmation.

Haemolysis is reported to result in a significant fall in measured insulin concentrations.^{42–44} Insulin-degrading enzyme (EC 3.4.22.11) is widely distributed in various tissues, including red blood cells, and shows a specificity for insulin

and transforming growth factor- α , with little activity for proinsulin, IGF-1 and glucagon. In addition to degrading endogenous insulin it has been suggested that the enzyme may degrade the iodinated insulin tracer used in radioimmunoassays.^{45,46} Even a small degree of haemolysis will invalidate results.

Proinsulin

There is little in the literature regarding the stability of this hormone. Smith and colleagues,⁴⁰ using an immunometric assay for proinsulin, found significant differences in concentrations between heparinized plasma and serum from matched samples, the former giving higher results. The effects of different sample types should be determined for all assays. No changes in measured proinsulin concentrations on repeated freezing (-20°C) and thawing of sample were found.⁴⁰ Whether these findings are applicable to other immunoassays, tube types and subjects awaits confirmation. The effect of haemolysis on proinsulin(s) concentrations has not been reported, although the insulin-degrading enzyme (EC 3.4.22.11) shows low activity for proinsulin.^{45,46}

C-peptide

C-peptide immunoreactivity in samples is reported to decline with time, although the extent of this appears variable and dependent on the antiserum/assay used. This may reflect *in vitro* production of C-peptide fragments, which react differently with different antisera, and may also explain the observation that the apparent stability of samples, standards and quality control materials may differ when measured with the same assay, although a variety of temperatures (-10°C , -20°C and -25°C) have been used to investigate the stability of frozen samples.^{13,47-49} The effect of repeated freeze/thaw cycles on measured C-peptide also appears to be variable, with some authors reporting no effect, although instability on storage at -20°C was found.⁵⁰ Other workers report small changes (at -20°C)⁴⁰ or changes varying from 2-26% of the measured concentration (at -10°C).¹³ These effects may also be assay-dependent.^{47,51} Faber *et al.*⁴⁷ showed a 28% decline in measured C-peptide on storage for 4 weeks at -25°C when using a particular antiserum in the assay but they found no significant changes when the assay was based on three other antisera. The effects of storage at -70°C were not investigated in these studies and it remains to be

demonstrated whether storage of samples (as well as standards and quality control materials) at this temperature is necessary for optimum stability. It is recommended that samples for C-peptide should be separated and stored frozen immediately. To avoid repeated freezing and thawing, the sample should be stored in aliquots.

Urinary C-peptide has been reported to be stable if frozen at -20°C for between 2 days and 1 year⁵² but the number of samples studied was very small ($n=3-6$) and (in view of the finding in serum that reported stability is assay-dependent) it is suggested that the stability of urinary C-peptide requires further investigation.

Haemolysis is reported not to affect C-peptide concentrations⁴⁴ although, as the accuracy of C-peptide assays is very dependent on the antibody used, it is suggested that this requires confirmation, particularly for assays based on non-isotopic labels.

As summarized above, it is suggested that C-peptide is less stable than insulin and proinsulin(s). In view of the fact that both insulin and C-peptide are required in the investigation of hypoglycaemia for diagnostic purposes, it would seem prudent for laboratories to recommend immediate transport of the sample, on ice, to the laboratory, with immediate separation and storage of the sample as frozen aliquots. This should ensure that the sample is suitable for the assay of C-peptide as well as insulin. A fluoride-oxalate sample for the measurement of glucose by the laboratory should also be sent.

METHODOLOGY

There are three main methods for analysis: bioassay, chromatographic techniques and immunoassay. The first two will be considered only briefly as they are unlikely to be used by the routine diagnostic laboratory.

Bioassay

Although bioassays for insulin measure net insulin-like activity, they have significant disadvantages and are not used by routine clinical laboratories. The bioassays, both *in vivo* and *in vitro*, may be affected by insulin antagonists and insulin-like substances. All the assay systems are lengthy, and relatively imprecise and insensitive. Examples of *in vitro* bioassays are the increase in glycogen of isolated rat diaphragm in glucose medium and glucose uptake by the rat epididymal fat pad. Radioreceptor assays are based on the competition between radiolabelled and

unlabelled hormone for binding to a specific receptor such as human placental membranes, rat erythrocyte membrane, fat or liver cells. Bioassays may be used by the appropriate organisations for establishing insulin standards.⁵³

Chromatographic techniques

Chromatography and, more particularly, high-performance liquid chromatography (HPLC) have been widely used for the analysis of insulin and its precursors in the pharmaceutical industry (where great sensitivity is generally not required) and for research studies. For the analysis of insulin and proinsulin(s) in biological fluids it is necessary to pretreat the sample and also to use a non-specific immunoassay to detect separated fractions. Thus, affinity chromatography followed by reverse phase HPLC and RIA of separated fractions has been used.^{54,55} Linde *et al.*⁵⁶ described a method using immunoextraction of 10 mL of serum followed by lyophilization of the sample prior to reverse-phase HPLC and analysis of separated fractions by immunoassay. These techniques are not suited to the routine laboratory since they require large sample volumes and are often laborious and technically demanding. Assay performance data such as recovery and precision are limited. In experienced hands, these methods are useful for the validation of immunoassays, for research purposes and for the analysis of samples from patients being investigated for the surreptitious injection of insulin where different animal insulins may be present.⁵⁷⁻⁶⁰

More recently, stable isotope dilution mass spectrometry assays have been described for insulin, proinsulin and C-peptide.^{14,61} After addition of a known amount of a labelled analogue of the hormone to the sample there follows an extraction and purification step using affinity chromatography and reverse-phase HPLC. The hormone and its analogue are then detected by mass spectrometry with an electrospray ion source. Comparison with immunoassay suggests that there may be some overestimation of insulin by immunoassays, particularly at low concentrations. In addition, isotope dilution assay has demonstrated that the 33-residue of C-peptide (comprising two additional amino acids, lysine and arginine at the C terminus after incomplete processing of proinsulin) makes up 4–10% of the 31-residue C-peptide concentration, although it was undetectable at a 31-residue C-peptide concentration of less than 1600 pmol/L.

Immunoassays

Insulin

Since the description of the principles of RIA for insulin by Yalow and Berson in 1959² the technique has been widely applied and developed. Much of the early literature is based on the classic RIA using polyclonal antisera, most usually with second antibody precipitation.

With the identification of proinsulin as the biosynthetic precursor for insulin by Steiner and Oyer³ it became apparent that RIA using polyclonal antisera would detect both insulin and proinsulin to varying degrees. Figures for proinsulin cross-reactivity in insulin RIAs vary from 38% to 100%.^{28,29} A number of studies have now shown elevations of proinsulin and its intermediates in type 2 diabetes mellitus and in impaired glucose tolerance,^{28-30,62-64} suggesting that it is not possible to assess insulin status in these conditions with non-specific insulin assays. Immunometric assays, in which an antibody is labelled, were developed in order to increase the sensitivity of assays and (in the two-site format) to increase specificity.^{65,66} In the mid-1970s Kohler and Milstein^{67,68} described the principles for the production of monoclonal antibodies in large amounts. This meant that it was possible to use monoclonal antibodies as analytical reagents in excess, as required by the immunometric format. The specificity of assays can be tailored by careful selection of antibodies to particular epitopes of interest. Consideration of assay design suggests that immunometric assays will be more sensitive than competitive assays, although the affinity of the antibodies used is critical. More recently, monoclonal antibodies with defined specificity have been used in combinations that exclude insulin precursors from reaction in a subsequent immunoassay for insulin.⁶⁹ However, the sample size of 100 µL and a detection limit of 11 pmol/L (expressed as mean concentration + 3 standard deviations of the zero signal) may be limiting.

Subsequently, there has been a proliferation of assays published in the literature, using a variety of labels, particularly non-isotopic, and with a variety of solid phases. The number of commercially available assays has grown and there are a number of areas of particular concern. Firstly, many published methods give insufficient detail of assay performance to allow intelligent interpretation of results. For earlier work, the lack of availability of proinsulin(s) to assess cross-reactivity was a problem. With the availability of biosynthetic proinsulin and partially processed

proinsulins,⁷⁰ improvements in assay performance data are to be expected. Secondly, both standardization and quality assessment remain areas where there is lack of consensus and appropriate materials. These will be considered in later sections. Thirdly, assay sensitivity (especially for the RIAs) may be limiting, although there have been some improvements with immunometric assays. Measurement of the low concentrations of insulin found in the fasting state and in hypoglycaemia due to non-pancreatic causes may be compromised by imprecision. The report from the American Diabetes Association Task Force on standardization of insulin assays⁷¹ noted the poor precision of some assays at low and high concentrations and commented that better precision was obtained with two-site assays. As with all immunoassays, other characteristics of assay performance such as linearity and recovery are important but there are few published data, and users of commercial assays are strongly recommended to seek this information from manufacturers.

There have been many in-house assays described for insulin and variability in their performance may explain the different findings of many studies⁹ and the disparate values for plasma insulin concentrations in fasting subjects. Details of some recently developed insulin assays

are shown in Table 1,⁷²⁻⁸¹ the majority of which are immunometric assays.

Many insulin assays have become commercially available in recent years and details of these (for human insulin) are shown in Table 2.⁸²⁻⁸⁹ Assays for insulin from other species are available. The manufacturers provided the data quoted. It should be noted that the information may not be complete and comparison is complicated because of the different methods of data presentation used by different manufacturers, particularly with regard to the units used for reporting results and the different conversion factors suggested. Some assays are available for research use only. The components of the assay – antisera and labelled hormone – may be available separately. Where possible the Tables in this review contain reference to published evaluations. These may well be limited to publication in abstract form and it should be noted that comparison with other methods may give limited information. Thus, for example, several comparisons are made with the widely available IMx assay (Abbott Diagnostics, see Appendix) without acknowledgement that this assay has been reported as being calibrated to 92.5% of the International Reference Preparation (IRP) 66/304.⁸⁴ It is to be hoped that the development of isotope dilution mass spectrometry assays^{14,61}

TABLE 1. *Characteristics of published insulin assays*

Method	Antibodies	Sensitivity* (pmol/L)	Cross-reactivity** (%)	Reference
Immunofluorometric	Mab	Zero + 2 SD 1.7	PI = 0	72
Enzymeimmunoassay	Pab	t-test of zero replicates 0.14	ns	73
Immunoenzymometric	Mab	2–3 times background 1.3	Some with PI	74
Immunoenzymometric	Mab	Zero + 3 SD 25	PI = 33	75
Immunoenzymometric	Mab	Zero + 2.5 SD 13.4	PI = 20	76
Immunoradiometric	Mab	Zero + 3 SD 2.3	PI = 5.3 32,33 split PI = 5.0 65,66 split PI = 62	77
Immunoenzymometric	Mab	Zero + 3 SD 0.8	ns	78
Immunoenzymometric	Mab	ns	PI = nd	79
Immunoenzymometric	Mab	Zero + 3 SD 5.0	PI = nd des 31,32 PI = nd des 64,65 PI = 63	80
Immunofluorometric	Mab	90% confidence interval 3.9 mU/L	PI = nd	81

Mab = monoclonal antibody; Pab = polyclonal antibody; PI = proinsulin; ns = not stated; nd = not detected.

*Definition of sensitivity used and result; **cross-reactivity with human hormones.

TABLE 2. Commercial assays for insulin

Label	Separation	Sample size (μ L)	Standard	Sensitivity*	Cross-reactivity** (%)	Company
<i>Competitive assays</i>						
¹²⁵ I	PEG	100	IRP 66/304	ns 2 μ IU/mL	PI = 15 C-peptide < 0.01	Biodata
	2nd Ab	100	IRP 66/304	ns 3.6 μ IU/mL	PI = 40	CIS
	Coated tube	100	IRP 66/304	ns 2.0 μ IU/mL	ns	CIS
	Coated tube	200	IRP 66/304	Zero - 2 SD 1.2-1.5 μ IU/mL	C-peptide = nd	DPC
	2nd Ab	100	Porcine	ns 1.3 μ IU/mL	des 64,65 PI = 68 des 31,32 PI = 50	DSL
	2nd Ab	200	IRP 66/304	ns 2.4 μ IU/mL	PI = 30 C-peptide = 0.01	Incstar
	PEG/2nd Ab	100	ns	ns 2 μ IU/mL	PI < 0.2 des 31,32 PI < 0.2 des 64,65 PI = 76	Linco ⁸²
	2nd Ab	100	ns	ns 0.2 μ IU/mL	PI = 6.0 des 31,32 PI = 6.0 des 64,65 PI = 78	Linco ('Ultra sensitive assay')
	2nd Ab	100	Not international standard	95% B/B ₀ 3.6 μ IU/mL	PI = 40 C-peptide = nd	Medgenix
	2nd Ab	100	IRP 66/304	ns < 2 μ IU/mL	PI = 41 C-peptide < 0.18	Pharmacia
	2nd Ab	100	IRP 66/304	ns 2.5 μ IU/mL	C-peptide < 0.01	Sorin
<i>Non-competitive assays</i>						
¹²⁵ I	Coated tube	50	IRP 66/304	ns 0.2 μ IU/mL	PI < 0.0001 32,33 split PI < 0.0004 65,66 split PI = 100 C-peptide < 0.003	Sanofi Diagnostics Pasteur ⁸³
	Coated tube	50	IRP 66/304	ns 1 μ IU/mL	PI = nd	Medgenix
	Coated tube	50	IRP 66/304	ns 0.3 μ IU/mL	PI = 32.7 C-peptide = 6.2	Sorin
ALP/methylumbelliferyl phosphate	Magnetic particles	150	IRP 66/304 (92.5%)	ns 1 μ IU/mL	PI = 0.005 32,33 split PI } None significant 65,66 split PI } C-peptide }	Abbott Laboratories (IMx analyser) ⁸⁴⁻⁸⁶
HRP/ABTS	Bead	100	IRP 66/304	ns < 3 μ IU/mL	PI = 40 C-peptide = nd	Boehringer, Enzymun (Manual option available)
HRP/TMB	mtp	25	IRP 66/304	Zero + 2.5 SD 3 pmol/L	PI = 0.3 32,33 split PI = 0.3 65,66 split PI = 45 C-peptide = nd	DAKO
HRP/TMB	mtp	50	IRP 66/304	Zero + 2 SD 0.15 μ IU/mL	PI = nd	Medgenix
HRP/TMB	mtp	25	IRP 66/304	Zero + 2 SD < 1 mU/L	PI < 0.01 C-peptide < 0.01	Mercodia
Electrochemiluminescence	para-magnetic beads	200	ns	Minimal detectable concentration 22 pg/mL	ns	Origen ⁸⁷

Continued

TABLE 2. Continued

Label	Separation	Sample size (μ L)	Standard	Sensitivity*	Cross-reactivity** (%)	Company
<i>Non-competitive assays (continued)</i>						
Chemi-luminescent	Magnetic particle	20	IRP 66/304	Zero + 2 SD 0.21 pmol/L	PI < 0.03	Sanofi Diagnostics Pasteur ^{88,89}
ALP/methyl umbelliferone	Magnetic particle	ns	ns	Zero + 2 SD 2.0 μ U/mL	C-peptide = nd	Tosoh (AIA series)
Chemi-luminescent	mtp	25	IRP 66/304	Zero + 2 SD 0.25 mU/L	PI = 1.2 des 31,32 split PI = 0.8 des 64,65 split PI = 44	Molecular Light Technology
Eu chelate	mtp	50	IRP 66/304	Zero + 2.5 SD 0.5 μ U/mL	PI = 0.1 32,33 split PI = 0.4 des 64,65 split PI = 66	EG and G Wallac

Ab = antibody; ABTS = 2,2-azino-di-[3,ethylbenzthiazoline sulphonate (6)]; ALP = alkaline phosphatase; B/B₀ = binding/zero binding; HRP = horse radish peroxidase; IRP = International Reference Preparation; mtp = microtitreplate; nd = not detected; ns = not stated; PEG = polyethylene glycol; PI = proinsulin; TMB = tetramethylbenzidine. *Definition of sensitivity used and result; **cross-reactivity with human hormones.

TABLE 3. Characteristics of published proinsulin assays: competitive assays

Sensitivity* (pmol/L)	Cross-reactivity** (%)	Fasting concentrations in control subjects (pmol/L)	Reference
Zero - 2 SD 0.01	ns	n = 24 Mean = 9.0 Range = 0-24.0	95
Zero - 2 SD 4.0	32,33 split PI = 0.1 65,66 split PI = 100	n = 23 Mean = 15 Range = 7-24	98
ns 1.2	des 31,32 split PI = 100 des 64,65 split PI = 100	n = 38 Median = 4.1 95% range < 1.2-13	99, 100
Zero - 2 SD 1 fmol/tube	32,33 split PI = 3.0 65,66 split PI = 100	n = 13 Mean = 2.7 SD = 1.5	36
Zero - 2 SD 3.0	des 31,32 split PI = 164 des 64,65 split PI = 151	n = 10 Mean = 10.7 SEM = 1.5	101
Zero - 2 SD 3.0	32,33 split PI = 100 65,66 split PI = 100	n = 9 Mean = 11 SEM = 2.0	28
ns 9.0	ns	n = 12 Median = 8.0 Interquartile range = 1.5-27	102
Zero - 2 SD 2.0	32,33 split PI = 66 65,66 split PI < 1.0	n = 25 Mean = 5.8 SD = 3.3	29
ns 3.5	des 31,32 split PI = 44 des 64,65 split PI < 1.0	n = 15 Mean = 3.6 SEM = 0.5	103 Antibody 168Ab
Zero - 2 SD 4.0	ns	n = 17 Mean = 6.7 SD = 1.7	104

Ab = antibody; ns = not stated; PI = proinsulin; SEM = standard error of the mean. *Definition of sensitivity used and level; **cross-reactivity with human hormones.

will lead to improvements in the assessment of the accuracy of immunoassays.

It is anticipated that further developments in assay technology will increase the number of automated assays. A homogeneous assay has been described,⁹⁰ as has a thermometric enzyme-linked immunoassay.⁹¹ Whether improvements in assay sensitivity and comparability will follow remains to be established.

Proinsulin

Assays for proinsulin make particular demands of sensitivity and specificity. Proinsulin and its partially processed forms contain the structures of insulin and C-peptide and as these are present in much higher concentrations than proinsulin there may be significant cross-reactivity.

Early assays for proinsulin were based on chromatographic separation followed by RIA for C-peptide and/or insulin in the separated fractions.^{92,93} An assay based on the degradation of insulin in serum by an insulin-specific protease followed by immunoassay for proinsulin-like material has been shown to be unreliable.⁹⁴

The first direct RIA for human proinsulin in serum was based on extraction of insulin and

proinsulin with an insulin antibody immuno-adsorbent followed by RIA of the C-peptide. Using this assay⁹⁵ it was found that proinsulin comprises approximately 20% of the basal immunoreactive insulin in control subjects. An indirect two-site immunoradiometric assay⁹⁶ confirmed these findings.

However, it became apparent, with the availability of biosynthetic proinsulin and its partially processed forms, that earlier assays may have overestimated proinsulin concentrations through incorrect calibration and cross-reactivity with the partially processed proinsulins.⁹⁷ Details of competitive and immunometric assays described in the literature are given in Tables 3 and 4.^{23,28,29,36,64,77,78,95,98-110} Antibodies used in these assays may be available from the sources quoted or others, e.g. Mab A6/3B1²⁷ (BioChem Immunosystems), Mab PH5/PH6¹¹¹ (Scottish Antibody Production Unit); see Appendix for manufacturers' details. Details (supplied by the manufacturers) of commercially available assays are shown in Table 5. Although increasingly used for research purposes, there are few detailed, published evaluations of such assays' performance. The interested reader

TABLE 4. Characteristics of published proinsulin assays: non-competitive assays

Sensitivity* (pmol/L)	Cross-reactivity** (%)	Fasting concentrations in control subjects (pmol/L)	Reference
Zero + 3 SD 1.8	32,33 split PI < 1.0 65,66 split PI = 66	n = 8 Mean = 2.4 SEM = 0.4	64, 77
Zero + 3 SD 0.8	32,33 split PI = 2.3	ns	78
Zero + 2.5 SD 0.1	32,33 split PI = 200 65,66 split PI = 68	n = 16 Mean = 4.73 SD = 2.92	105
Zero + 3 SD 0.25	des 31,32 split PI = 65 des 64,65 split PI = 99	n = 38 Median = 4.0 Range = 2.1-12.6	106
Zero + 2.5 SD 0.017	ns	ns	107
Zero + 2 SD 0.4	des 31,32 split PI = nd des 64,65 split PI = 75	n = 30 Mean = 3.4 Range = 1.0-9.1	108
Zero + 3 SD 1.0	des 31,32 split PI = nd des 64,65 split PI = 75	n = 22 Mean = 1.2	109
Zero + 2 SD 0.2	Insulin = nd C-peptide = nd	n = 132 Median = 9.0 Range = 3-25	23
Zero + 3 SD 0.2	Insulin = nd C-peptide = nd des 31,32 split PI = nd des 64,65 split PI = nd	n = 20 Median = 2.7 Range = 1.1-6.9	110

nd = not detected; ns = not stated; PI = proinsulin; SEM = standard error of the mean.

*Definition of sensitivity used and level; **cross-reactivity with human hormones.

should seek further information from manufacturers. Because of the limited availability of the partially processed forms of proinsulin, not all assays have been characterized in terms of their cross-reactivities with these prohormones. It should be noted that cross-reactivity with the des 32,33 split proinsulin is variable but significant. It is this form of partially processed proinsulin which is present in detectable concentrations in the circulation and hence is of greater significance in terms of cross-reactivity in proinsulin assays. An immunoassay for this form has been reported,⁷⁷ which (with the concurrent measurement of intact proinsulin) allows for correction for the cross-reactivity of intact proinsulin in the assay. The antibodies 3B1 and PEP001 were used⁷⁷ although other combinations such as 3B1 and CPT3F11 (DAKO Diagnostics) may yield improvements in assay performance. An assay using the antibodies 3B1 and PEP001 with enzyme amplification as the detection system is commercially available for the measurement of total proinsulins (see Table 5).

C-peptide

Significant advances, particularly in terms of automation and alternatives to isotopic labels, have been made in the measurement of C-peptide in serum and urine since earlier reviews.^{113,114} Despite the greater choice of commercially available assays many of the analytical issues, such as problems of standardization and stability of the hormone, are still relevant.

There are a number of technical issues peculiar to the development of immunoassays for C-peptide. It has a low molecular weight (3021 Da for human C-peptide) and is therefore a poor immunogen. It also lacks a tyrosine residue, which must therefore be introduced for iodination. There are significant species differences in structure and therefore the human form should be used for standards, immunogen, quality control materials and label production. As mentioned previously, there is heterogeneity in the circulating forms of C-peptide and the different forms may react to a greater or lesser

TABLE 5. Commercial assays for proinsulin(s)

Label	Separation	Sample size (μL)	Standard	Sensitivity* (pmol/L)	Cross-reactivity** (%)	Company
<i>Competitive assays</i>						
¹²⁵ I	PEG/2nd Ab	200	ns	ns 2.0	Insulin <0.1 des 31,32 PI = 95 des 64,65 PI <0.1 C-peptide <0.1	Linco
<i>Non-competitive assays</i>						
ALP enzyme amplification	mtp	50	IRR 84/611	ns	Insulin = nd	DAKO (intact proinsulin)
ALP enzyme amplification	mtp	50	IRR 84/611	ns	Insulin = nd	DAKO (total proinsulin)
HRP/TMB	mtp	100	IRR 84/611	ns	Insulin = nd C-peptide = nd des 31,32 PI <1.0 des 64,65 PI = 53.2	DRG ¹¹²
HRP/TMB	mtp	50	IRR 84/611	Zero + 2SD 0.5 pmol/L	Insulin <0.03 C-peptide <0.006	Mercodia
Chemi-luminescent	mtp	50	IRR 84/611	Zero + 2SD 0.2	Insulin = nd C-peptide = nd des 31,32 split PI = 1.4 des 64,65 split PI = 63	Molecular Light Technology (intact proinsulin)
Chemi-luminescent	mtp	50	IRR 84/611	Zero + 2SD 0.35	Insulin = 2.2 C-peptide = nd des 31,32 split PI = 100 des 64,65 split PI = 1	Molecular Light Technology (total proinsulin)

ALP = alkaline phosphatase; HRP = horse radish peroxidase; IRR = International Reference Reagent; mtp = microtitreplate; nd = not detected; ns = not stated; PEG = polyethylene glycol; PI = proinsulin; 2ndAb = second antibody; TMB = tetramethylbenzidine. *Definition of sensitivity used and level; **cross-reactivity with human hormones.

extent with different antisera, and behave differently to the labelled C-peptide and standard, leading to non-parallelism.^{13,115} Non-specific displacement of tracer by serum proteins can lead to falsely elevated values^{47,116,117} and there is poor comparability of results between

assays, with a variety of reference ranges being quoted.^{48,50,118,119} Many assays show significant cross-reactivity with intact proinsulin (and presumably with partially processed proinsulins). Removal of these cross-reactants by gel chromatography or immunopurification is not

TABLE 6. Commercial assays for C-peptide

Label	Separation	Sample size (μL)	Standard	Sensitivity*	Cross-reactivity** (%)	Company
<i>Competitive assays</i>						
¹²⁵ I	PEG	100	ns	Zero - 2 SD 0.1 ng/mL	Insulin nd up to 100 000 ng/mL PI = 32	Biodata
	Coated tube	100	IRR 84/510	ns 0.025 pmol/mL	Insulin = 0.01 PI = 13	CIS
	2nd Ab/PEG	25	ns	Zero - 2 SD 0.05-0.22 ng/mL	Insulin = nd PI ~ 18	DPC
	2nd Ab	50	ns	ns 0.01 ng/mL	Insulin = nd PI = 4.0	DSL
	PEG/2nd Ab	100	IRR 84/510	ns 0.04 ng/mL	Insulin < 0.1 PI = 10 32,33 split PI = 24 des 64,65 PI = 24 65,66 split PI = 17 des 31,32 PI = 26	Guildhay
	2nd Ab	100	Synthetic C-peptide	ns 0.05 ng/mL	Insulin = 0.01 PI < 4.0	Incstar
	2nd Ab	100	ns	ns 0.1 ng/mL	Insulin = nd PI < 4.0	Linco
	Coated tube	100	IRR 84/510	ns 0.03 pmol/L	PI = 22	Medgenix
	Coated tube	100	IRR 84/510	ns 0.025 pmol/L	Insulin < 0.01 PI = 10	Sorin
	HRP	mtp	100	ns 95% B/B ₀ 0.05 ng/mL	Insulin = nd PI = 0.5	DRG
	ALP	Bead	50	ns	Insulin = nd	DPC
	HRP	mtp	100	IRR 84/510	0.3 ng/mL Zero - 2 SD 0.014 pmol/mL	PI = nd PI = 12.5 Immulite ¹²¹ Medgenix
<i>Non-competitive assays</i>						
HRP	mtp	25	ns	Zero + 2.5 SD 17 pmol/L	Insulin = nd PI = 63 32,33 split PI = 75 des 31,32 PI = 82 65,66 split PI = 71 des 64,65 PI = 87	DAKO
ALP/4-methyl umbelliferone	Magnetic particle	20	IRR 84/510	Zero + 2 SD 0.2 ng/mL	Insulin = 0.005 PI = 69	Tosoh (AIA series) ¹²²
HRP/TMB	mtp	25	IRR 84/510	Zero + 3 SD 15 pmol/L	Insulin < 0.1 PI = 100	Mercodia
Eu chelate	mtp	25	Synthetic C-peptide	Zero + 2.5 SD 0.015 ng/mL	Insulin = nd PI = 51 32,33 split PI = 34.9 des 64,65 split PI = 91.7	EG and G Wallac

ALP = alkaline phosphatase; B/B₀ = binding/zero binding; HRP = horse radish peroxidase; IRR = International Reference Reagent; mtp = microtitreplate; nd = not detected; ns = not stated; PEG = polyethylene glycol; PI = proinsulin; 2ndAb = second antibody; SD = standard deviation; TMB = tetramethylbenzidine. *Definition of sensitivity used and level; **cross-reactivity with human hormones.

practicable for the routine diagnostic laboratory. It should be noted that there are several orders of magnitude difference in the circulating concentrations of C-peptide (nmol/L) and proinsulins (pmol/L). However, whilst cross-reactivity might be minimal in a normal fasting individual, care should be taken when interpreting results from non-fasting individuals or in situations where the circulating concentrations of proinsulin(s) may be elevated.

Two-site immunometric assays for C-peptide have been described using both mono- and polyclonal antibodies. Kao, Taylor and Hesser¹²⁰ developed an immunochemiluminometric assay using two affinity purified polyclonal antibodies from different animals and with a significant reduction in assay incubation time to 4 h (some RIAs for C-peptide involve an overnight incubation). It is not clear whether the use of immunometric assays will reduce some of the analytical problems associated with the instability of the hormone or whether assays of differing specificities may lead to greater variability of reference ranges and lack of comparability.

There are a number of automated systems now available that have assays for C-peptide, and details of commercial assays are shown in Table 6.^{121,122}

There have been numerous studies measuring C-peptide in urine (e.g. references 39, 123–5). Generally, assay protocols suggest sample dilution, although there has been little published on assay validation and all the above-mentioned factors are likely to affect the performance of urine assays.

STANDARDIZATION

The standardization of immunoassays for insulin, proinsulin and C-peptide remains a problem, despite wider availability of large quantities of insulin and proinsulin through recombinant DNA technology. This becomes obvious through a close scrutiny of, for example, the variety of reference ranges quoted in the literature,⁹ although each analyte has its own difficulties.

Insulin

In early assays the Fourth International Standard for Insulin, for Bioassay, a mixture of bovine and porcine insulin, was used as a standard.⁵³ The First International Reference Preparation for insulin (coded 66/304) is a purified preparation of human insulin and has no defined

content in terms of mass. It has been widely used as a standard, both primary and secondary, for insulin immunoassays, and the majority of commercially available insulin assays are stated to be calibrated against this IRP. It should be noted that in a survey of diagnostic companies for this article, calibration of kits was not always given as 100% of this IRP. The First International Standard for human insulin (coded 83/500)¹²⁶ is a purified human preparation and has a quoted potency of 26 000 U/g. Using these data and a molecular weight of 5808 Da for this standard would give a conversion factor of 1 mU = 6.6 pmol. From amino acid analysis of this preparation it has been suggested^{127,128} that a conversion factor of 1 mU = 6.0 pmol should be used. However, this factor may not be applicable to IRP 66/304, which does not have a defined content in terms of mass. In a survey of British laboratories, conversion factors (mU/L to pmol/L) ranged from 6.0–7.5 irrespective of the standard used.¹²⁹ At the time of writing both the above standards are available from the National Institute of Biological Standards and Controls (NIBSC), Potters Bar, UK. The conversion factor used must depend on the standard used in the assay and for commercial assays confirmation of this should be sought from the manufacturer. Where results for insulin, proinsulin(s) and C-peptide are reported it is more usual to quote these in molar terms, particularly if a ratio of proinsulin(s) to insulin is to be calculated.

Recently, the American Diabetes Association⁷¹ organised a task force on standardization of insulin assays. It assessed comparability of insulin measurements between laboratories using 17 insulin assays. Significant variability in insulin results was obtained for the distributed samples which (surprisingly) was not reduced by the use of a common standard or curve fitting procedure. In addition, when a lyophilized standard requiring reconstitution and dilution was distributed to participating laboratories the results indicated that the laboratory preparation of the standard contributed to the variability in results.

The task force noted that the relationship of the assays to each other remained the same and therefore, comparability of results would be obtained if all laboratories related their results by regression to one method. They suggested that assessment and certification of insulin assays should follow three steps: (a) assessment of internal performance, (b) determination of

external comparability and (c) recertification. No currently available proficiency and certification program was judged to be adequate and the task force recommended that a central laboratory be established to provide certification. They suggested that laboratories should be requested to make available, for publication, details of assay performance, quoting the regression factor to the central laboratory assay for their assay. In addition, acceptable performance for an assay was defined by the task force as:

- Accuracy: comparison to within 5% of the 'gold standard' throughout the working range. The 'gold standard' was not specified.
- Recovery: $\pm 15\%$ throughout the working range
- Precision: the coefficient of variation should not exceed 15% throughout the range of the calibration curve
- Specificity: should be reported
- Linearity: observed concentration should be within $\pm 15\%$ of the expected value

Wider discussion of these recommendations amongst professional groups and the diagnostics industry will be important. Such full details of assay performance are rarely published and laboratories and manufacturers should be encouraged to provide such data. These recommendations were aimed at laboratories involved in clinical research and epidemiological studies but undoubtedly have implications for clinical diagnostic laboratories.

Proinsulin

Early assays were calibrated against pancreatic extracts of human proinsulin and inevitably the presence of proteolytically cleaved proinsulins in the preparation will have led to inaccuracies of calibration.⁹⁷ Biosynthetic proinsulin is now used to calibrate most assays and human proinsulin is available as an IRR (coded 84/611) from the NIBSC.

C-peptide

Biosynthetic preparations of C-peptide are available but standardization remains a problem. The first IRR was coded 84/510. The standard coded 76/561 is a synthetic 64-formyl lysine C-peptide and includes four extra basic amino acids, two at each end. The presence of formyl lysine at position 64 is reported to improve stability and solubility in aqueous solution. Even using this common standard, variability of results between different assays was still obtained,¹¹⁷ and parallelism with

other standards in all assay systems studied was not always obtained.

QUALITY ASSURANCE

External

There are external quality assessment programs available for insulin and C-peptide [College of American Pathologists Series 2 Ligand Proficiency Testing Program (USA), and schemes organised by Dr D Teale, Guildford (UK) and Murex, Dartford (UK)]. Most schemes are limited by the small number of participating laboratories and the difficulty in defining adequate performance and in distributing clinically relevant samples. There are no official schemes for proinsulin at the time of writing although some specialist laboratories within the UK participate in a voluntary sample exchange scheme.

Internal

Some commercially available assays for insulin, proinsulin and C-peptide include materials with quoted ranges for internal quality assessment. Generally, the concentration limits are wide and the nature of the material is not stated. Quality control materials suitable for these assays should be selected with care. In particular, the nature of the (added) insulin, proinsulin or C-peptide should be stated and the effect of preservatives on assay performance should be examined. There are as yet no suitable commercially available materials to act as quality control samples for proinsulin assays, and the use of samples which have been assayed previously can be helpful. Samples to which proinsulin is added to appropriate concentrations can also be used. The instability of C-peptide means that care should be taken in the preparation and storage of quality control materials.

INTERFERENCES

As discussed previously, the main issues of specificity for these immunoassays are the cross-reactivity of the prohormones in assays for insulin and *vice versa*. One of the main difficulties in assessing cross-reactivity is the lack of availability of the partially processed forms of proinsulin.

Immunoassays for proinsulin(s) and insulin are potentially subject to interferents commonly reported for other immunoassays, such as heterophilic antibodies, rheumatoid factor, complement, etc. Boscato and Stuart¹³⁰ reported that

non-analyte antibody binding substances could be found in approximately 40% of serum samples. This could be overcome by the addition of excess non-specific immunoglobulin from an appropriate species, although it should be noted that the amount added routinely might not always be sufficient to block the interference. Whilst it may be possible to identify such interference in subjects being investigated during the course of a dynamic function test, a falsely elevated insulin concentration in a patient with hypoglycaemia may not be easy to identify and may result in an incorrect diagnosis of insulinoma and inappropriate treatment. It is strongly recommended that insulin, C-peptide and pro-insulin should all be measured as any inconsistency in the results might indicate interference in an assay.

Insulin antibodies: anti-insulin antibodies and insulin autoantibodies

Circulating antibodies to insulin in the human can occur (a) as an immune response to treatment with exogenous insulin, particularly non-human insulin (anti-insulin antibodies), (b) in the development of type I diabetes mellitus or (c) as part of an autoimmune process or following therapy with certain thiol drugs (insulin autoantibodies). Anti-insulin antibodies tend to have a higher affinity for insulin than do the autoantibodies. Whilst there may be a small number of cases where these antibodies are of clinical relevance, causing autoimmune hypoglycaemia,¹³¹ controversy surrounds their general importance and this is compounded by significant differences in the assay methodology used to detect them.^{132,133} Antibodies to proinsulin have also been described.¹³⁴

Anti-insulin antibodies in patients' samples have been shown to interfere in RIAs for insulin leading to either overestimation or underestimation depending on the separation system used. There are numerous published methods for measuring total and free insulin in the presence of antibodies by, for example, using polyethylene glycol (PEG) precipitation or acid-alcohol extraction. These methods are critically dependent on specific sample collection and assay conditions¹³⁵⁻¹³⁷ and the accuracy of their results needs to be confirmed by independent assay.

The effect of insulin antibodies on reagent excess or immunometric assays is not clear. Whilst the relative affinities of monoclonal antibodies used in some of these assays might

be sufficient to overcome the established equilibrium between insulin and autoantibody, this has not been found to be the case in assays for thyroglobulin where measurement of the protein may be needed in patients with antithyroglobulin antibodies. Autoantibodies to thyroglobulin have been shown to interfere in both RIAs and immunometric assays.¹³⁸ There has been little work on the effect of anti-insulin antibodies in immunometric assays. Sapin¹³⁹ demonstrated a reduction in measured insulin in PEG-treated serum from 31 serum samples containing anti-insulin antibodies. This effect was shown for both a double-antibody RIA (Sanofi Pasteur) and for an immunometric assay (IMx, Abbott Diagnostics), suggesting that the insulin antibodies caused an increase in measured insulin in both assays. The author suggests that, for the RIA, an increase in measured insulin due to insulin antibodies is due to labelled insulin binding to the autoantibody but not being precipitated on addition of a second antibody reagent. It was suggested that, for the immunometric assay, there was at least partial recognition of the antibody-bound insulin by the antibodies used in the assay. However, control serum samples not containing antibodies to insulin were not included in this experiment and further work is required to establish the importance of these effects. Andersen *et al.*¹⁴⁰ found good agreement between 'free' insulin measurements and those by immunometric assay in samples where the measured insulin binding (i.e. insulin antibodies) was less than 25%. However, above this level there was no clear relationship between the two assays, suggesting that there might be some interference in the immunometric assay.

BIOLOGICAL VARIATION AND REFERENCE INTERVALS

The control of glucose and intermediary metabolism is complex, involving insulin and the counter-regulatory hormones glucagon and adrenaline and (to a lesser extent) cortisol and growth hormone.²¹ Insulin is secreted by the β cells of the pancreas in a pulsatile fashion¹⁴¹ and is secreted with C-peptide in equimolar quantities into the portal vein. Insulin is taken up by the liver to a large and variable extent, in contrast to C-peptide which undergoes minimal hepatic extraction. Peripheral concentrations of C-peptide may, therefore, more accurately reflect β cell insulin secretion rates. Insulin has

a half-life of approximately 4 min, proinsulin of 30 min and C-peptide of 33 min in non-diabetic, healthy individuals.¹⁴² C-peptide is excreted mainly by the kidney and hence circulating concentrations are elevated in renal disease.

It cannot be over-emphasized that for routine diagnostic use in the investigation of hypoglycaemia the most important concept is whether the measured hormone concentration is appropriate for the measured plasma glucose concentration. The definition of hypoglycaemia itself has been surrounded by controversy. It is possible to define hypoglycaemia in terms of (a) the range of glucose concentrations found when healthy volunteers fast for a defined period of time, i.e. statistically, (b) the range of glucose concentrations found in patients found subsequently to have an insulinoma, (c) the range of glucose concentrations at which there is a defined physiological/physical response, i.e., function, and (d) a symptomatic definition based on the presence of symptoms of the autonomic response or of neuroglycopenia.¹⁹ The plasma glucose definition chosen for hypoglycaemia may then affect that for the hormonal response. In the presence of demonstrable hypoglycaemia Marks and Teale²⁰ suggest that the plasma/serum insulin, C-peptide and proinsulin concentrations should be < 30 pmol/L, < 300 pmol/L and < 20 pmol/L, respectively, whilst Service²¹ quotes figures for the interpretation of a 72-h fast of insulin concentration < 36 pmol/L (non-specific assay), C-peptide < 200 pmol/L and proinsulin < 5.0 pmol/L, with a glucose concentration < 2.22 mmol/L. These figures can only be used as guidelines as assay methodology will have a significant influence.

For research purposes, and for the investigation of the syndromes of insulin resistance, it is possible to define reference intervals for a given population, either fasting or during certain procedures. It is clearly recognized that for all three hormones there is a significant variability in the reported ranges due to the definition of the population selected for study, assay methodology and standardization, the protocols used for the dynamic function tests used, and the sample type used. The following should be considered:

1. Age

Samples collected from cord blood show measurable concentrations of insulin, proinsulin and 32,33 split proinsulin and there is evidence that the insulin/glucose relationship is different

in neonates.^{26,27} There is an increase in insulin resistance at puberty.¹⁴³ Glucose intolerance is prevalent in the elderly and manifests itself with an increased postprandial glucose concentration and insulin response. A number of factors may contribute, such as physical inactivity, decreased lean body mass, decreased insulin secretion and increased insulin resistance. Even allowing for obesity and fitness it appears that there is still an effect of ageing when 60–92 year olds are compared with younger subjects,¹⁴⁴ although it is unusual to quote age-specific reference ranges.

2. Body mass index

There is a clear relationship between fasting insulin concentration, and the insulin response to a glucose load, and the degree of obesity. Elevations of circulating concentrations of insulin, proinsulin and C-peptide are more strongly associated with abdominal than with peripheral obesity^{99,145,146} and obesity is a risk factor for the development of type 2 diabetes. It is nevertheless unusual for reference ranges to be quoted for diagnostic purposes for different categories of body mass index, although published studies will control for obesity.

3. Gender

Although there are differences in body mass index and insulin sensitivity between men and women, it is not usual to quote separate reference ranges for diagnostic purposes. A decrease in insulin sensitivity has been demonstrated during the luteal phase of the menstrual cycle, associated with high concentrations of progesterone¹⁴⁷ and studies in women taking progestogens have confirmed this. It is not usual to quote separated reference ranges for the different stages of the menstrual cycle.

Similarly, a significant increase in insulin resistance is well documented in pregnancy, with increases in insulin and proinsulin concentration relative to glucose.¹⁴⁸

4. Sample collection conditions

Tissue uptake of glucose/insulin leads to an arterio-venous difference in measured insulin. For research purposes, particularly for glucose clamp techniques, it is recommended that arterial samples should be collected by hand warming and retrograde cannulation.¹⁴⁹

5. Other conditions

Many conditions other than diabetes mellitus and pancreatic tumours can affect glucose homeostasis and hence circulating concentrations of

these hormones. Thus, changes in these hormones have been described in conditions associated with glucose intolerance, such as cystic fibrosis and acromegaly. Defects in thyroid disease, cirrhosis and renal disease have also been noted,^{150,151} and the effect on circulating concentrations of C-peptide is important diagnostically.

There is significant variation in the reference ranges quoted for fasting concentrations of insulin, proinsulin and C-peptide, and there are clear differences due to assay methodology. Most research studies will use age-, gender- and body-mass-index-matched controls. However, reference ranges for fasting concentrations of these hormones in subjects with normal glucose tolerance (demonstrated by a normal oral glucose tolerance test) rarely take these factors into account. Care should be taken in deriving ranges from published data using the control group as numbers of subjects may be small and insufficient detail of statistical techniques may be given. Each laboratory should establish its own reference ranges and the following are given for guidance only: fasting plasma insulin, 18–90 pmol/L,¹⁵² (statistical definition not given); fasting plasma C-peptide, 180–740 pmol/L¹¹⁹ and 310–430 pmol/L¹⁰⁴ (both C-peptide ranges defined as mean \pm 2 standard deviations).

Concentrations for proinsulin in fasting control subjects are shown in Tables 3 and 4. Data for normoglycaemic subjects using isotope dilution analysis have been published and require confirmation.¹⁴

Dynamic function tests

It is beyond the scope of this review to consider in any detail the multitude of tests used to investigate β -cell function (insulin secretion) and insulin sensitivity (inversely related to resistance). These methods are primarily used for research purposes and a variety of protocols, even for a single test, have been used.^{153–159} The estimation of insulin sensitivity can be based on indirect methods (action of endogenous insulin) or direct methods (action of exogenous insulin).¹⁵⁷ The former tests include (a) homeostasis model assessment (HOMA) (from fasting insulin and glucose data), (b) insulin and glucose responses to the oral glucose tolerance test, (c) the intravenous glucose tolerance test, (d) the 'minimal model' developed from the intravenous glucose tolerance test with mathematical modelling and (e) continuous infusion of glucose

with model assessment (CIGMA).^{153,158,159} Direct measures of insulin sensitivity include the insulin suppression test, the euglycaemic hyperinsulinaemic clamp and the insulin tolerance test.¹⁵⁵ Estimates of insulin secretion have been made using the oral glucose tolerance test, the short intravenous glucose tolerance test, the minimal model, the hyperglycaemic clamp and CIGMA.^{154,159} It is becoming clear that seemingly minor changes in technique may cause significant changes in results. Research studies will be based on comparison to a defined control population and comparisons will be valid as long as a standard technique is used throughout. These tests are labour intensive and require skill and trained personnel. They should only be carried out in specialist centres under medical supervision. One significant component contributing to the accuracy of the results will be the type of insulin assay used and its specificity. For those tests based on the measurement of endogenous insulin in patients who may have an elevation of proinsulin(s) of up to 50% of total insulin immunoreactivity, the estimation of insulin using a non-specific assay may well overestimate insulin and hence insulin resistance. Much of the literature is based on the use of non-specific assays and these techniques need to be validated using specific assays. To date, the main use of these techniques has been in the investigation of the pathogenesis of diabetes mellitus. With the development of drug therapies which increase insulin sensitivity it is possible that the simpler techniques for estimating insulin sensitivity *in vivo* (e.g. HOMA) may become valuable in predicting which patients would benefit from such therapy. If so, laboratories offering a routine insulin assay service may need to consider the use of a specific assay for such purposes.

It is only in rare situations that dynamic function tests are required for diagnostic purposes. The C-peptide suppression test has been used for the investigation of hypoglycaemia and involves the measurement of C-peptide during insulin-induced hypoglycaemia. Use of this test is no longer recommended because of the dangers of the procedure and variability of the response, which depends on gender, age and body mass index.¹⁶⁰ The C-peptide response to glucagon has been used to determine whether patients with diabetes may need treatment with insulin or in cases where there is diagnostic confusion regarding the type of diabetes.¹⁶¹

Selective venous catheterization with measurement of hormone concentrations to localize insulin-secreting tumours has been advocated but generally gives poor results.²⁰

SAFETY

Immunoassays are the most commonly used assays in clinical laboratories for the measurement of insulin, proinsulin and C-peptide, and where isotopic end-points are employed the most important safety issues relate to the handling of ¹²⁵I. Appropriate safety precautions should be taken. For assays with non-isotopic end-points users are advised to consult the manufacturers' data sheets and Control of Substances Hazardous to Health (COSHH) information. Care should be taken in the storage and preparation of solutions of insulin not only because of its hypoglycaemic effect but also because of its potential abuse as an anabolic agent.

AUTOMATION

As for all immunoassays, many of the steps in immunoassays for insulin, proinsulin(s) and C-peptide can be simplified by the use of automatic x-y-z pipetter/dilutors, plate washers and appropriate curve-fitting computer programs. Where reagents are bought commercially the main limitation may well be the dead volume of the equipment and the small volume of reagent supplied. Care should be taken to avoid contamination and carry-over of concentrated solutions of, for example, antibody conjugate, and washing of equipment should be stringent. Some laboratory detergents may inhibit the enzyme activity of some detection systems and the quality of the distilled/deionized water used should be monitored. Several automated systems with these assays are now available and are indicated in Tables 2 and 6. Their use may improve throughput but issues of calibration and specificity should be considered.

COST

The number of laboratories offering these assays for diagnostic purposes is likely to be small, as the incidence of hypoglycaemia (excluding insulin-treated diabetics) is small. The cost of maintaining these assays and the associated expertise is not insignificant, and typical prices in the UK for a single sample range from £15 for C-peptide to £30 for proinsulin. To minimize

sample transport, freeze/thawing and to avoid delay in diagnosis it is preferable that a laboratory should offer both insulin and C-peptide and have access to a proinsulin assay. The measurement of proinsulin may be particularly important if a specific insulin assay has been used for the initial investigations.

CONCLUSIONS

It is clear that a laboratory must determine the main clinical requirements for the assays that it will be offering. For those involved in diabetes research there is a need for assays that are specific for insulin, intact proinsulin, partially processed or total proinsulins and for C-peptide. Assays for insulin and proinsulin(s) should have the ability to measure precisely concentrations below 1.0 pmol/L. Those laboratories whose main interest is in the investigation of hypoglycaemia have a more difficult choice to make. Unless all assays are offered there is the potential for missing an insulinoma that secretes mainly proinsulin if a very specific insulin assay is used. The pathological relevance of the relative secretion of insulin to proinsulin and their circulating concentrations in patients with pancreatic islet cell tumours is not yet known. Whichever assays are used, their sensitivities and specificities should be well documented and their limitations recognized. It is to be hoped that, with the wider availability of recombinant hormones and the partially processed proinsulins, there will be improvements in calibration and assay comparability. Full details of assay performance should be published.

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APPENDIX

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Biostat (Biodata)
Pepper Road
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CIS (UK) Ltd
Dowding House
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DAKO Diagnostics Ltd
Denmark House
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Diagnostic Products (DPC) Ltd
Glyn Rhonwy
Llanberis
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Fax. +44 (0)1286 871802

DRG (IDS Ltd)
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Tyne and Wear NE35 9PD, UK
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DSL (Metra Biosystems)
Forestry House
Haseley Trading Estate
Stadhampton Road
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Eurodiagnostica
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Fax. +44 (0)202 660020

Mercodia (Diagenics) Ltd
Freeport NG6287
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Medgenix (Lifescreeen, Appligene, Oncor) Ltd
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Igen Inc
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564 *Clark*

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